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CDX-1 and CDX-2 are expressed in human colonic mucosa and are down-regulated in patients with Hirschsprung's disease associated enterocolitis

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Abstract

Caudal type homeobox gene-1 and -2 (CDX-1 and CDX-2), homologues of the *Drosophila* homeobox gene caudal, encode transcription factors in endoderm derived tissues of the intestine. CDX genes control proliferation and differentiation of intestinal mucosal cells and colon cancer cells. Hirschsprung's Disease (HD) or congenital intestinal aganglionosis, a major developmental anomaly of intestine, which causes functional intestinal obstruction, is frequently associated with enterocolitis. Aetiology of HD-associated enterocolitis (HDEC) remains obscure. Reduction of gut mucosal enteroendocrine cells, and inefficient transfer of the secretory immunoglobulin A across the gut mucosal cell were shown to be associated with enterocolitis in HD patients suggesting that mucosa may directly involve in the pathophysiology of HDEC. This study aims to ascertain whether the CDX-1 and CDX-2 genes, that control the proliferation and differentiation of mucosal cells, play a role in HDEC. Using semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR) and in situ hybridisation, we analysed the expression of CDX-1 and CDX-2 genes in colon specimens of normal controls, necrotising enterocolitis (NEC) infants, and HD patients with and without enterocolitis. We showed for the first time that CDX-1 and CDX-2 genes were expressed in the colonic mucosal epithelium in normal, NEC and in HD infants. However, the expressions of both genes were reduced in patients with HDEC. Our findings suggest that reduced expression of CDX-1 and CDX-2 genes in mucosa may be associated with the development of HDEC.

Keywords: Human colon; Caudal type homeobox gene

1. Introduction

CDX-1 and CDX-2, members of the caudal-type homeobox gene family, are transcription factors that are expressed early in the intestines of human and rodent [1]. Cdx-2 mRNA was first detectable in endoderm and mesenchyme at the posterior gut in 8.5 days post coitum (d.p.c.) mouse embryos and was then progressively confined to the intestinal endoderm. Temporal and spatial expression patterns of Cdx-2 in the small intestine and colon parallel the morphogenesis of the definitive intestine [2]. The expression profile of Cdx-1 mRNA was different from Cdx-2 in that it was not detected until 14 d.p.c.

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where it could be found in the endoderm of the developing gut [3–5]. In the intestinal epithelium, Cdx-1 mRNA and protein were essentially confined to the crypts, but Cdx-2 mRNA and protein showed complex differential distributions. In the small intestine and distal colon, Cdx-2 mRNA was found predominantly in the crypts. However, the protein was most abundant in the differentiated cells that had already migrated up to the tip of the crypts. In the proximal colon, Cdx-2 mRNA and protein were evenly distributed from the bottom of the crypts to the differentiated epithelial cells at the tip. In mutant animal analysis, it was found that homozygous Cdx-1 and heterozygous Cdx-2 knockout mice frequently develop dysplastic lesions, metaplasia and homeotic transformation of endoderm to a rostral phenotype in the colon [6–9]. Therefore, Cdx-1 and Cdx-2 genes have been suggested to be involved in the regulation of the proliferation and differentiation of intestinal epithelial cells [7,10,11].

In human small intestine and colon, CDX-1 protein was localised in the nuclei of epithelial cells. Reduced levels of CDX-1 and CDX-2 mRNA were reported in colorectal cancer using reverse transcription–polymerase chain reaction (RT–PCR) and Northern blot analysis in human [5,12]. Truncating mutation of CDX-2 genes was reported in human colorectal cancers [11,13,14]. CDX-1 protein was shown to inhibit the proliferation of rat intestinal epithelial cells IEC6 by down-regulation of cyclin-D [15]. Cdx-1 is the direct transcriptional target of the Wnt signalling pathway [16], and Wnt signalling in gut requires the binding of the Tcf4/β-catenin complex to the Cdx-1 gene promoter, which induces the expression of Cdx-1. Abnormal Wnt and β-catenin signalling has been implicated in the carcinogenesis in colon of carcinomas, hepatocellular carcinomas and melanomas [17,18]. These findings are in line with the regulatory role of CDX-1 and CDX-2 in cellular proliferation, differentiation.

Hirschsprung's disease (HD), a major anomaly of the enteric nervous system (ENS), is characterised by the absence of intramural ganglion cells and the presence of hypertrophic nerve trunks in the distal intestine that results in functional intestinal obstruction. The pathogenesis of HD remains poorly understood. Recent studies of HD showed that the *RET*, *GDNF*, *EDN3*, *EDNRB* and *SOX10* genes were implicated in HD and mutations in these genes accounted for about 20% of the patients [19]. Enterocolitis is a serious complication of HD with a variable incidence of 6–58% and remains the major cause of morbidity and mortality in patients with HD [20,21]. Histologically, it is characterised by an acute inflammatory infiltrate into the crypts and mucosa of the intestinal epithelium in both the aganglionic and ganglionic segments. Speculation on the aetiology of HD-associated enterocolitis (HDEC) includes colonic stasis resulting from functional intestinal obstruction [22], bacteria infection [23], mucosal ischaemia [24], defects in the amount of immunoglobulin A [25] and hypersensitivity reaction [26], enteroendocrine cell deficiency [27]. Piebald-lethal mouse, a mouse model of HD, has missense mutations in the *ednrb* genes and frequently develops systemic sepsis caused by enteric organism [28,29]. It has been postulated that the loss of mucosal barrier integrity in the distal aganglionic bowel allows the translocation of enteric organism and causes systemic sepsis in piebald-lethal mouse. Intestinal mucosal epithelium is continuously renewed and replenished from stem cells that are located in intestinal crypts. Renewal and replenishing of mucosal epithelial cells is essential for the intestine to repair damage of mucosa and prevent bacterial infection and inflammation. Therefore, loss of mucosal integrity as a consequence of defective renewal and replenish of stem cells could be an initial event in the development of enterocolitis. While HD may be caused by mutations in a variety of genes, at present it is not clear whether there is a common molecular basis for HD-associated enterocolitis. We hypothesise that the CDX genes may be involved in maintaining the integrity of the intestinal epithelium by direct involvement in the control of epithelial cell proliferation and differentiation. In this study, we aim to determine whether CDX-1 and CDX-2 genes are involved in the pathology of HD-associated enterocolitis by investigating the tissue-specific pattern of the distribution of CDX-1 and CDX-2 transcripts in colon from non-HD and HD patients with and without enterocolitis. In this study, we show that the CDX-1 and CDX-2 genes are expressed in the epithelium of normal colon, and further provide the first evidence that reduced expression of these genes is associated with HD-associated enterocolitis.

2. Materials and methods

2.1. Tissue preparation for RNA extraction and sections

Resected specimens of colon were collected from 11 HD patients and analysis of the rectal biopsy samples of these patients revealed an absence of ganglion cell. Haematoxylin–eosin staining, acetylcholinesterase histochemical staining on rectal biopsies of patients with HD performed by pathologists confirmed the diagnosis of HD in all eleven patients. The clinical diagnosis of enterocolitis was made on the basis of the presentation of diarrhea, fever and an distended abdomen. Age- and site-matched specimens of the distal colon from 12 non-HD patients (imperforate anus, $n=5$; rectal stricture, $n=1$; mesenteric cysts, $n=1$; necrotising enterocolitis, $n=5$) were included as controls. Control specimens were obtained from normal colon proximal to and well away from the diseased bowel; histology analysis of control specimens confirmed no dysganglionosis. Confirmation of enterocolitis in both patients with HD and those with necrotising enterocolitis (NEC) patients were made by histological analysis of resected bowels. Full-thickness specimens from the aganglionic, hypoganglionic and normoganglionic zones of bowel of each patient and the colons of normal controls were used for RNA extraction using Trizol (Life Technologies, Rockville, MD). For tissue sections, the specimens were fixed in 4% paraformaldehyde/PBS (phosphate-buffered saline; pH 7.2) and embedded in paraffin. Sections (6 μm thickness) were prepared on microtome and mounted onto 3-aminopropyl-triethoxy-silane (Sigma) coated slides, dried and baked at 60°C for 1 h.

2.2. Semi-quantitative RT–PCR analysis

Two μg of total RNA was applied for the synthesis of first strand cDNA using oligo(dT) and random hexamers as reverse primers (SuperScript Preamplification System, Life Technologies). Expressions of CDX-1, CDX-2 and β-actin genes were analysed using the PCR. PCR was performed in 50 μl of standard buffer containing primers (0.2 μM each), 2 μl of cDNA, 0.5 unit of AmpliTaq DNA polymerase (Life Technologies), $MgCl_2$ (3 mM), and dNTP (0.2 mM). Dimethyl sulphoxide (0.5%; v/v) was added to the reaction for the PCR of CDX-1 and CDX-2. After the initial denaturation process at 94°C for 3 min, PCR was performed for 30 and 35 cycles for β-actin gene and CDX genes, respectively, as follows: 1 min denaturation at 94°C; 1 min annealing (68°C for CDX-1 and β-actin, 70°C for CDX-2); 1 min extension at 72°C for each cycle. A final extension for 10 min at 72°C was added. As controls, PCRs were performed with the same set of RNAs but without the addition of reverse transcriptase to test for genomic contamination. Details of primers used for RT–PCR are shown in Table 1. Nucleotide positions of forward and reverse primers of CDX-1, CDX-2 and β-actin genes were numbered according to their mRNA sequences [11,12,30]. RT–PCR reactions of each sample were performed in duplicates. One-fifth of the PCR products was analysed by electrophoresis in a 2% agarose gel. Intensities of PCR bands were quantified and relative expression levels of CDX-1 and CDX-2 to β-actin were determined using the comparative method as described by Nicoletti and Sassy-Prigeut [31]. Means and standard error of the mean (S.E.M.) of the relative expression of CDX-1 and CDX-2 were determined for each group. Un-

Table 1
Primer sets for CDX-1, CDX-2 and β-actin genes

Gene	Primer sequence (5′–3′)	Nucleotide position	Annealing temp. (°C)
CDX-1	F, AGG ACA AGT ACC GCG TGGTCT A	553–1106	68
	R, CCT CTG AAC GTA TGG AGG AGG A		
CDX-2	F, GAA CCT GTG CGA GTG GAT GCG	275–941	70
	R, GGT CTA TGG CTG TTGG GTG GGA G		
β-actin	F, CCT CGC CTT TGC CGA TCC	8–618	68
	R, GGA TCT TGA GGT AGT CAG TC		

Nucleotide sequences of forward primers (F) and reverse primers (R) are shown.

paired *t*-test was performed to evaluate the significance of the differences between the relative expression levels of CDX-1 and CDX-2 in controls, NEC and various segments of patients with HD. The two-tailed *P* values of the comparison between the relative expression level of CDX genes of various groups were determined and difference was considered to be significant if the *P* value was less than 0.05.

2.3. Cloning of CDX-1 and CDX-2

RT–PCR products of CDX-1 and CDX-2 gene were cloned in pGEM-T Easy Vector (Promega). Nucleotide sequences of CDX-1 and CDX-2 clones were determined by Sanger's dideoxy chain termination method [32] using T7 sequencing kit (Amersham Pharmacia Biotech).

2.4. Immunohistochemistry

The endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 for 10 min. Antigen retrieval was performed by heating the slides in 10 mM citrate buffer (pH 6.0) at 95°C for 10 min. The sections were treated and incubated with primary and HRP-conjugated secondary antibodies. Anti-S100 (Clone 4C4.9; NeoMarkers; 1:100 dilution), and anti-Ret R5 (Nagoya University; 1:100 dilution) were used in this study. Negative controls were included in which the primary antibody was omitted. Antibody incubations were performed in PBST (PBS+0.1% Tween 20), supplemented with 10% horse serum. Primary antibody was incubated with sections at 4°C for 16 h. Incubation of secondary antibody was performed at 37°C for 1 h. Signals were visualised using strepABComplex/HRP kit (Dako) and 3,3′-diaminobenzidine tetrahydrochloride (Sigma, UK). Sections were counterstained with haematoxylin, dehydrated, cleared in xylene and mounted in DPX mountant (BDH).

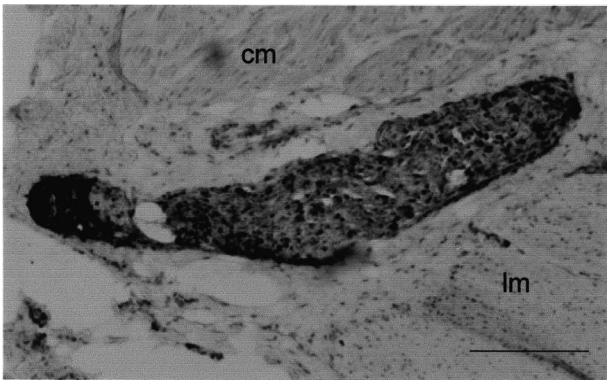

Fig. 1. S100 immunostaining of aganglionic colon of patient with HD. S100-immunoreactive thick nerve bundle is localised in the myenteric plexus. lm, longitudinal muscle; cm, circular muscle. Scale bar = 100 μm.

2.5. In situ hybridisation analysis

Single-stranded [^{35}S]UTP-labelled sense and antisense riboprobes were generated from linearised CDX-1 and CDX-2 clones. Synthesis of riboprobes, hybridisation, autoradiography and histological staining were performed as described previously [33]. In situ slides were exposed for 21 days. Photographs of in situ hybridisation were taken on an Axioplan 2 microscope (Carl Zeiss) fitted with a Sony digital camera under dark-field or bright-field illumination.

3. Results

3.1. Diagnosis of controls and HD patients

Using anti-Ret and anti-S100 antibodies, we

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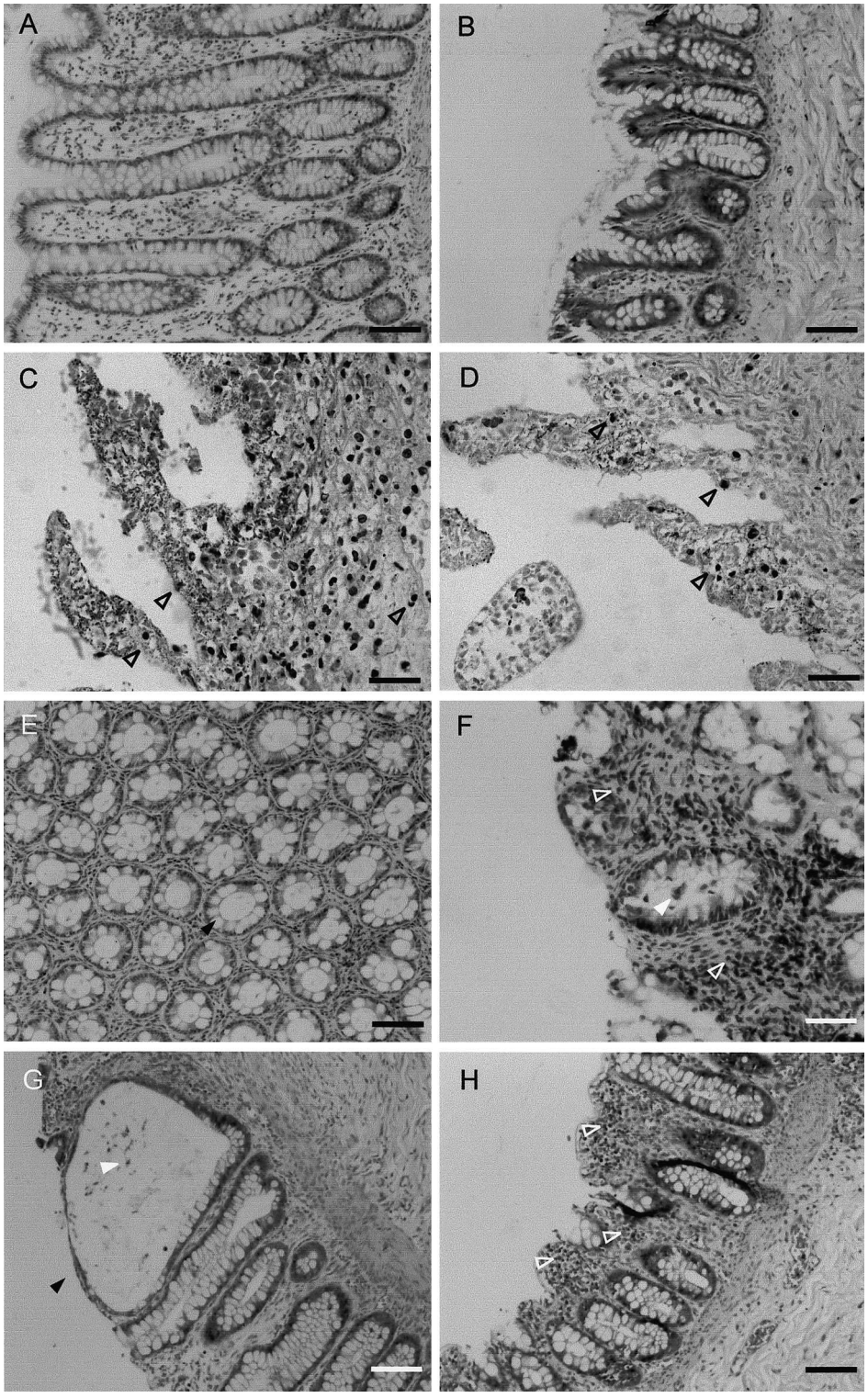
Fig. 2. Histological analysis of colon sections of non-HD control infants, infants with necrotising enterocolitis (NEC) and patients with HD. In the colon of a non-HD control infant (A), crypts are long and intact with no obvious enlargement. In a patient with NEC (B), the crypts are shorter but intact, and are slightly enlarged. Mucosal ulceration and lymphocytes infiltration in crypts (unfilled arrowhead) are noted in NEC samples (C,D). In HD patients with enterocolitis, slightly enlarged crypts (black arrowhead) in hypoganglionic colon of patient 4421 (E) and heavily enlarged crypts (black arrowhead) in the normoganglionic colon of patient 8117 (G) are frequently observed. Lymphocyte infiltration (white arrowhead) in the crypt is also noted in the aganglionic colon of patient 4421 (F) and in the normoganglionic colon of patient 8117 (G). Epithelial linings are severely damaged and disappeared in the aganglionic colon of patients 4421 (F) and 8117 (H). Lymphocytes are frequently found in the crypts (white arrowhead) and papilla (unfilled arrowhead). Scale bars: A,B,E,G,H = 100 μm; C,D,F = 50 μm.


A
B
C
D
E
F
G
H

showed in the aganglionic segment the absence of Ret immunopositive neuron (data not shown) and the presence of S100-immunopositive hypertrophic nerve trunks in the myenteric plexus which are typical features of aganglionic bowel of patients with HD (Fig. 1). The diagnosis was also confirmed by histochemical staining for acetylcholinesterase that showed intense staining in HD diseased colons (data not shown). The degrees of enterocolitis were graded in controls and all eleven patients with HD according to the histological grading system of Teitelbaum et al. [34]. In the control subjects without enterocolitis, no crypt dilation and cryptitis (grade 0) were found in the mucosa (Fig. 2A). In the controls with necrotising enterocolitis (NEC), slight dilated crypts were infrequently observed (grade I) and mucosa appeared shorter with intact epithelium in two NEC samples (Fig. 2B and data not shown), ulceration of mucosa with infiltrated lymphocytes were observed in three other NEC samples (grade IV) (Fig. 2C,D and data not shown). Diagnosis and clinical history of the controls and patients with HD are shown in Table 2. Among the 11 patients with HD, no remarkable changes were observed between segments of colon in the seven patients without enterocolitis, and specimens were graded as either grade 0 or grade I (data not shown). In the remaining four patients, various degrees of enterocolitis were observed in the specimens. In one patient, the majority of crypts showed dilation with mucin retention (grade II) while isolated crypt abscesses were observed in the aganglionic segment (Fig. 2E,F). In another three patients, severe dilated crypt and multiple crypt abscesses (grade IV) were frequently observed in the hypoganglionic segment (Fig. 2G and data not shown). Mucosal ulceration and infiltrated lymphocytes into the lamina propria and crypt lumen (grade IV) were commonly found in the aganglionic segment (Fig. 2H and data not shown).

Table 2
Clinical data of the patients with HD and control

	Case no.	Age	Diagnosis	Colostomy
Control	5499	1 month	Rectal stricture	+
	5918	4 months	Imperforate anus	+
	6641	12 months	Imperforate anus	+
	NC1	72 months	Mesenteric cysts	−
	B206	2 days	Imperforate anus	−
	B240	2.3 months	Imperforate anus	−
	6665	30 months	Imperforate anus	+
NEC	6775	4 months	Necrotising enterocolitis (grade I)	+
	6763	12 months	Necrotising enterocolitis (grade I)	+
	B238	9 days	Necrotising enterocolitis (grade IV)	−
	B231	16 days	Necrotising enterocolitis (grade IV)	−
	B230	5 days	Necrotising enterocolitis (grade IV)	−
HD	4554	4 days	Short HD	+
	4668	8 days	Short HD	+
	4845	24 days	Short HD	−
	5585	30 days	Short HD	+
	6297	30 days	Rectal HD	−
	B211	2.5 months	Short HD	+
	B241	2 months	Short HD	+
HDEC	8117	1 days	Short HD, enterocolitis (grade IV)	+
	4421	18 days	Short HD, enterocolitis (grade II)	+
	8444	24 days	Short HD, enterocolitis (grade IV)	+
	B100	2.6 months	Short HD, enterocolitis (grade IV)	+

Details including age, diagnosis of HD and non-HD controls are shown. Patients and controls that have or have not received colostomy are indicated as '+' and '−', respectively.

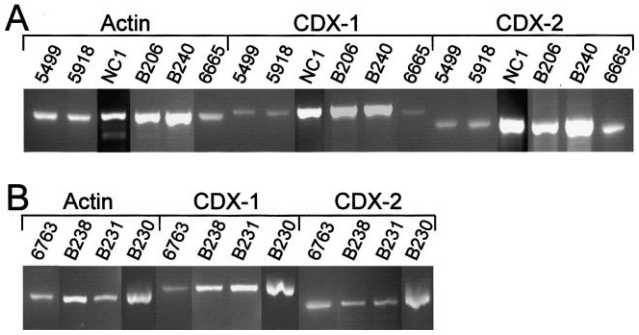


Fig. 3. RT–PCR analysis of CDX-1 and CDX-2 gene expressions in the colons of non-HD controls and patients with NEC. CDX-1 and CDX-2 genes are co-expressed in the colons of non-HD control infants (A) and in patients with NEC (B). Identity numbers of the control infants are shown above the lanes.

3.2. *CDX-1 and CDX-2 transcripts are co-distributed in the colonic mucosal epithelium*

To determine the tissue-specific pattern of distribution of transcripts of CDX-1 and CDX-2 genes in the colon, we first detect transcripts of CDX-1 and CDX-2 in the distal colon of infants using RT–PCR. CDX-1 and CDX-2 mRNAs were detected in the colon of infants aged between 2 days and 30 months (Fig. 3). Using RT–PCR analysis, relative levels of expression of CDX-1 and CDX-2 genes versus β-actin was determined in colon of non-HD infants and in patients with NEC. Ratios of CDX-1/β-actin and CDX-2/β-actin in the colons of non-HD infants were determined to be 0.59 ± 0.23 and 1.19 ± 0.35 (mean ± S.E.M.) respectively whereas the ratios of CDX-1/β-actin and CDX-2/β-actin in the colons of patients with NEC were 1.28 ± 0.24 and 0.86 ± 0.18 respectively. Relative expression levels of CDX-1 and CDX-2 gene were not significantly different between colons of non-HD infants and patients with NEC (Table 3). RT–PCR products of CDX-1 and CDX-2 genes were cloned and nucleotide sequences were determined to be identical to the published sequences of CDX-1 and CDX-2 genes. No amplified product was obtained in negative controls in which reverse transcriptase was omitted. This indicates that the amplicons observed in our samples were not PCR products of the contaminating genomic DNA (data not shown).

In order to determine whether CDX-1 and CDX-2 mRNAs were confined to a particular cell lineage or compartment within the colon, in situ hybridisation was performed in the colons of the controls. In the distal colons of controls, CDX-1 and CDX-2 transcripts were present in the mucosal epithelial cells but not in the lamina propria or muscle layer (Fig. 4A–D). Differential distribution of CDX-1 transcript was observed in the mucosal epithelium in which staining was more intense in the undifferentiated cells at the bottom of crypts (Fig. 4E). Unlike CDX-1, CDX-2 transcript was more evenly distributed from the bottom to the tip of crypts (Fig. 4F). Sense probes of CDX-1 and CDX-2 genes showed weak and no specific background hybridisation signals (data not shown).

Table 3
Statistical analysis of expression of CDX

	CDX-1/β-actin (mean ± S.E.M.)	CDX-2/β-actin (mean ± S.E.M.)
Controls ($n=7$)	0.59 ± 0.23	1.19 ± 0.35
NEC ($n=5$)	1.28 ± 0.24 ($P=0.08$)[a]	0.86 ± 0.18 ($P=0.5$)[a]
HD ($n=7$)		
Normoganglionic segment	0.87 ± 0.22 ($P=0.47$)[a]	1.42 ± 0.61 ($P=0.74$)[a]
Hypoganglionic segment	0.51 ± 0.08 ($P=0.21$)[b]	0.75 ± 0.23 ($P=0.37$)[b]
Aganglionic segment	0.92 ± 0.20 ($P=0.86$)[b]	1.03 ± 0.29 ($P=0.6$)[b]

Unpaired *t*-test was performed to evaluate the significance of the difference of the relative expression levels of CDX-1 and CDX-2 in controls, NEC and various segments of HD patients.

[a]Two-tailed *P* values of the comparison of the relative expression level of CDX genes in NEC and the normoganglionic segment of HD patients with the controls ($P < 0.05$ is considered to be significant).

[b]Two-tailed *P* values of the comparison of the relative expression level of CDX genes in NEC and hypoganglionic and aganglionic segments with normoganglionic segments of patients with HD ($P < 0.05$ is considered to be significant).

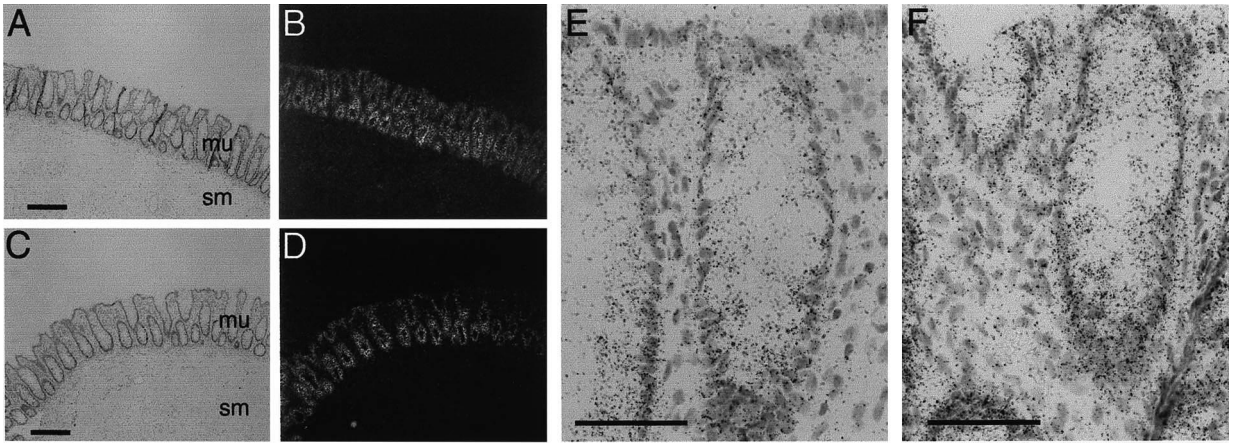


Fig. 4. In situ hybridisation analysis of CDX-1 and CDX-2 mRNAs in the colons of normal control infants. Transcript of CDX-1 (A,B,E) is localised to the colon epithelium with strongest hybridisation signal towards the bottom of the crypts. Transcript of CDX-2 (C,D,F) is localised to colonic epithelium and is more evenly distributed from the bottom to the tip of crypts. Photographs of the hybridised sections are taken under bright-field illumination (A,C,E,F) or dark-field illumination (B,D) to show the distribution of CDX-1 and CDX-2 mRNAs in colons. Scale bar = 100 μm.

3.3. *Down-regulation of CDX-1 and CDX-2 genes in the colons of patients with HD-associated enterocolitis*

To determine whether CDX-1 and CDX-2 genes were involved in the pathogenesis of HD-associated enterocolitis (HDEC), we used RT–PCR to compare the relative expression levels of CDX-1 and CDX-2 genes in the normoganglionic, hypoganglionic and aganglionic segments of the distal colon of HD patients with and without enterocolitis using RT–PCR. In the seven cases of HD without enterocolitis, expression levels of CDX-1 and CDX-2 genes were similar among the three segments (Fig. 5 and data not shown). The ratios of CDX-1/β-actin and CDX-2/β-actin were not significantly different between the various segments of patients with HD, Patients with NEC, or non-HD controls (Table 3). However, in patients with HDEC ($n = 4$), CDX-1 and CDX-2 gene expression levels are markedly reduced in the aganglionic and hypoganglionic segments than in normoganglionic segment (Fig. 5). RT–PCR bands of CDX-1 and CDX-2 were barely detectable in hypoganglionic and aganglionic segments in two patients with HDEC (patient 8117 and B100; Fig. 5). Our RT–PCR data indicated that both CDX-1 and CDX-2 genes were down-regulated in the hypoganglionic and aganglionic bowels of patients with HDEC where enterocolitis were obvious. The inten-

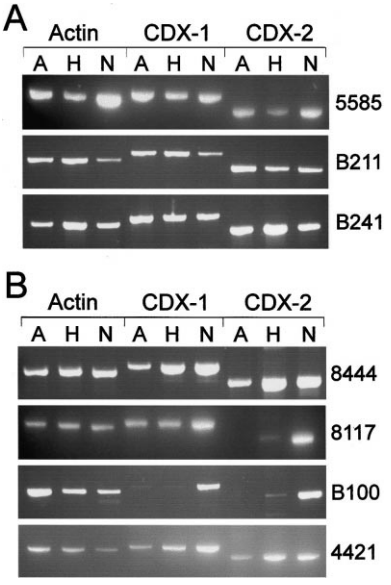


Fig. 5. Lower levels of expression of CDX-1 and CDX-2 in the aganglionic colons of patients with HDEC. In panel A, representative RT–PCR analysis results of patients with HD are shown. CDX1 and CDX2 genes are expressed at a similar level in different segments of patients with HD. In panel B, in patients with HDEC, expression of CDX1 and CDX2 genes is reduced in aganglionic (A) and hypoganglionic (H) segments compared to their levels in the normoganglionic (N) segments of patient's resected colon. Identity numbers of the patients are shown on the right.

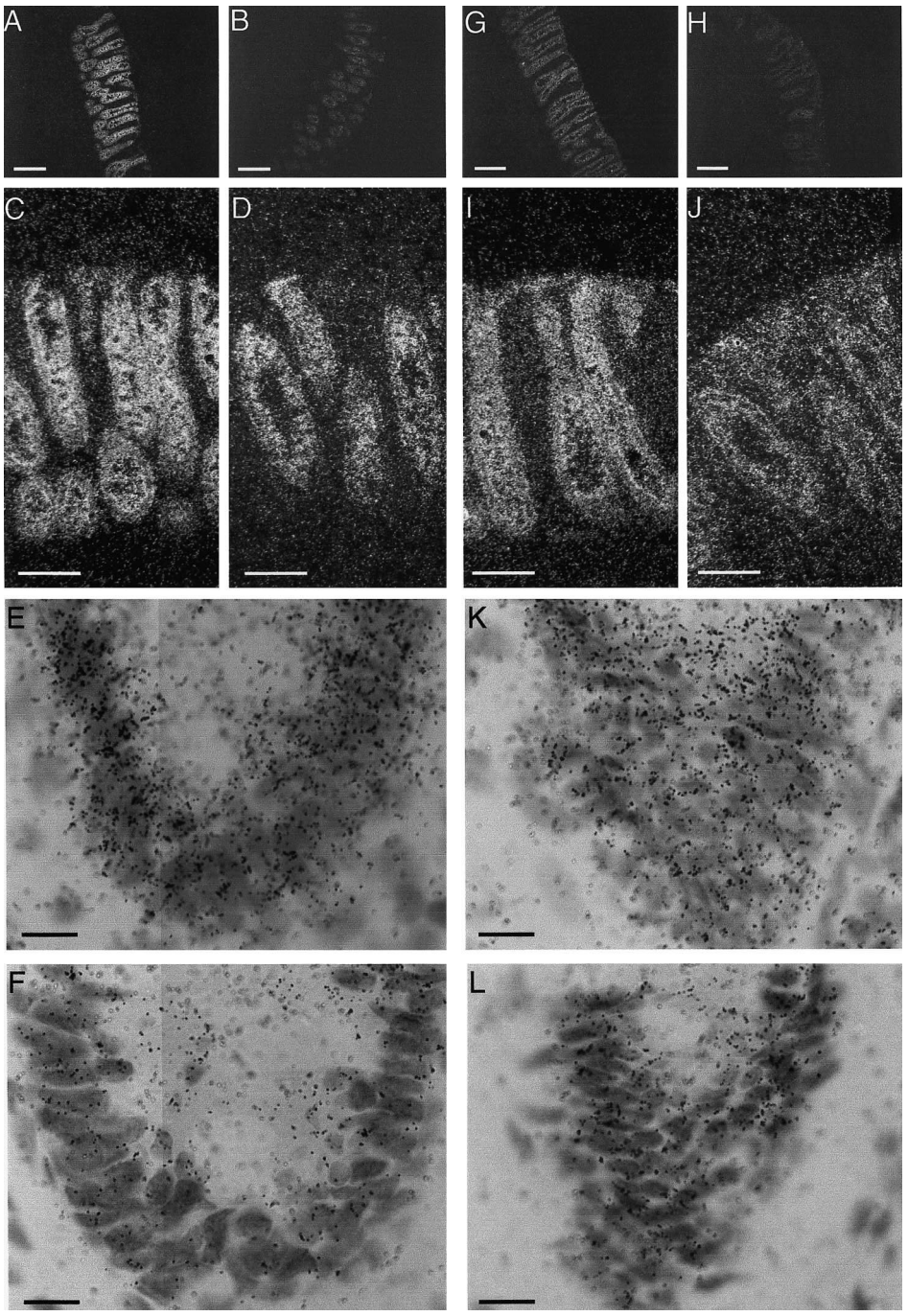


Fig. 6. In situ hybridisation analysis showed the reduction of CDX-1 and CDX-2 gene expressions in the aganglionic colon of patients with HD. Representative in situ hybridised colon sections of patients with HD are shown. Photographs of hybridised sections of the colon of patient 8444 are taken under dark-field illumination (A–D,G–J) or bright-field illumination (E,F,K,L). The level of CDX-1 transcript is lower in the aganglionic segment (B,D) than that in the normoganglionic segment (A,C). CDX-2 gene expression is down-regulated in the aganglionic segment (H,J) compared with the normoganglionic segment (G,I). Number of silver grains for the CDX-1 gene (E,F) and CDX-2 gene (K,L) in the mucosal epithelial cells are fewer in the aganglionic (F,L) than in the normoganglionic segments (E,K). Scale bars: A–D,G–J = 100 μm; E,F,K,L = 10 μm.

sities of the RT–PCR bands of CDX-1 and CDX-2 genes in the hypoganglionic and aganglionic bowels were too weak to be quantified in these two patients with HDEC. No statistical analysis was performed on the HDEC data because of insufficient quantification data in the HDEC group.

In patients with HD, in situ hybridisation detected CDX-1 and CDX-2 transcripts only in the mucosal epithelial cells (Fig. 6). In patients with HDEC, the levels of CDX-1 and CDX-2 gene expression in the mucosal epithelium were lower in aganglionic segments when compared with those of normoganglionic segments (Fig. 6A–D,G–J). The reduced levels of expression for CDX genes observed could be due to the fact that fewer CDX expressing cells were present in the epithelium and/or each of the epithelial cells was expressing lower levels of CDX genes. The intensity of silver grains on the sections was counted under bright field illumination. As illustrated in Fig. 6, the number of silver grains derived from the CDX-1 and CDX-2 probes found in each epithelial cell were fewer in the aganglionic segment than in the normoganglionic segments. This indicates that the epithelial cells of aganglionic segments expressed CDX-1 and CDX-2 genes at reduced levels (compare Fig. 6E,F,K,L). Mucosal ulceration was evident in aganglionic segments of HDEC patients (Fig. 2F) suggesting that overall, there were fewer epithelial cells surrounding the mucosa of aganglionic segments. Therefore, it was likely that the reductions in expression of CDX-1 and CDX-2 genes in mucosal epithelium in the aganglionic colons of patients with HDEC were the results of fewer cells in the mucosa; and these cells were expressing lower levels of both CDX-1 and CDX-2 genes.

4. Discussion

The present study aims at determining whether the homeobox genes CDX-1 and CDX-2 were involved in HD-associated enterocolitis (HDEC). We have analysed the expression of CDX-1 and CDX-2 genes in distal colon of normal control infants as well as patients with HD. Our results show that CDX-1 and CDX-2 genes are expressed in the mucosal epithelium of distal colons of infants aged between 2 days and 30 months. In patients with HDEC, the expression levels of CDX-1 and CDX-2 genes decrease in the aganglionic and hypoganglionic bowels.

4.1. Reduced expression of CDX genes in HD-associated enterocolitis

Three of our patients with HDEC who have undergone colostomy before the pull-through procedure have grade IV enterocolitis in aganglionic segments, suggesting that accumulation of stool is unlikely to be the main factor causing local inflammation of intestine. Instead, other cellular abnormalities, probably localised in epithelial layer, could be contributing to the susceptibility of inflammation.

Intestinal mucosal epithelium is continuously renewed and replenished from stem cells that are located in the intestinal crypts and give rise to absorptive enterocytes, enteroendocrine cells, mucus-secretion goblet cells and Paneth cells. Renewal and replenishing of mucosal epithelial cells is essential for the intestine to repair mucosa damage and prevent bacterial infection and inflammation. Failure to renew damaged mucosa could lead to an increased risk of developing enterocolitis. Our RT–PCR analysis revealed decreased expression of CDX genes in aganglionic and hypoganglionic segments in all four patients with HDEC. In situ hybridisation analysis clearly confirmed that expression of CDX genes decreased in individual epithelial cells. Using adjacent sections for in situ hybridisation, we have also investigated the expression of the glia cell-line derived factor family receptor α1 (GFRA1) gene in two of the three patients with HDEC. The GFRA1 gene expressed in the myenteric and submucosal plexuses of the intestine [35]. We found that the GFRA1 gene was differentially regulated in the myenteric and submucosal plexuses of hypoganglionic and aganglionic segments in one patient with HDEC; whereas expression of GFRA1 gene was not altered in the second patient with HDEC (V.C.H.L. and P.K.H.T., unpublished data). These findings confirmed that the down-regulation of CDX genes in the aganglionic bowels of all four patients with HDEC observed in this study was not due to artefacts of improper tissue processing that led to RNA degradation or general lower expression of genes in particular sections. Unlike the case with HDEC, five

of our controls have necrotising enterocolitis (NEC) but did not show reduced levels of CDX transcripts. This suggests that HDEC and NEC possess distinct aetiologies. Our results may suggest that the decreased expression of CDX-1 and CDX-2 genes is associated with the occurrence of HDEC. It has been shown that CDX-2 regulates the expression of cell adhesion molecules E-cadherin, integrin-β4, -β1, L1-CAM, and extracellular matrix molecule laminin-γ2, -α1 chain [1]. Interestingly, the migrating and differentiating cells along the crypt-villus axis show a concomitant increasing gradient of some of these molecules [36]. Reduced levels of expression of CDX genes in the patients' bowels may provoke changes in the compositions and properties of cell adhesion complexes and extracellular matrix (ECM) of the mucosa. Such changes may result in defective proliferation and differentiation of the mucosal epithelial cells and manifest as increased risk of inflammation.

4.2. Hirschsprung's disease genes and enterocolitis

In the present study, with the exception of the four patients with HDEC, none of the patients with HD showed reduction of CDX-1 and CDX-2 gene expression in aganglionic segments. It was shown that homozygous Cdx-1 and heterozygous Cdx-2 knockout mice did not develop defect in the enteric nervous system [7–9]. Our results suggest that CDX-1 and CDX-2 genes are not directly involved in HD and insufficient levels of CDX-1 and CDX-2 is not critical for enteric nervous system development. HD is caused by mutations in a variety of genes including the *RET*, *GDNF*, *EDN3*, *EDNRB* and *SOX10* genes and mutations in these genes accounted for about 20% of the patients [19], suggesting that novel HD genes remain to be identified. Mutations of the *RET* most frequently mutated gene in patients with HD, were identified in only four out of 57 cases of sporadic and familial HD (7%) [37]. This suggests that HD is a polygenic disease caused by the additive subclinical effects of more than one gene. Although the genetic defects of the patients with HDEC included in the present study are not defined, it is unlikely that they have the same genetic defect. The association of the down-regulation of CDX genes with the occurrence of enterocolitis in these patients with HDEC could be explained if the mutations of different HD genes affected the same signalling pathway for ENS development and the regulation of CDX genes expression. Mutations of such HD genes in these patients with HDEC caused defects in the same signalling pathway and resulted in colonic aganglionosis and down-regulation of CDX-1 and CDX-2 genes as well as increased risk of developing enterocolitis in these HDEC patients. Piebald-lethal mouse, a mouse model of HD, has missense mutations in the *ednrb* genes and frequently develops systemic sepsis caused by enteric organism [28,29]. It has been postulated that the systemic sepsis in piebald-lethal mouse is the consequence of the loss of mucosal barrier integrity in the distal aganglionic bowel. Our present findings suggest that the loss of mucosal integrity may represent a critical and common event in the aetiology of HDEC. Further study of the expressions of CDX genes in patients with HDEC is essential to determine whether the down-regulation of CDX-1 and CDX-2 genes is a common mechanism in all HDEC.

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